HUMAN CHORIONIC GONADOTROPIN VACCINES

BACKGROUND OF THE INVENTION

A promising approach in the effort to moderate population growth has been the use of immunogens directed against the hormones involved in reproductive physiology. It is believed that the administration of such immunogens to a mammal causes the mammal to generate specific antibodies which inactivate these hormones, thereby arresting their functions. At least six such immunogen formulations of human (h) reproductive hormones are now or have been in clinical trials: one against follicle-stimulating hormone (FSH) [Moudgal, N.R. et al., Prospectives in Primate Reproductive Biology, 297-306 (1991)]; two against gonadotropin-releasing hormone (GnRH) [Ladd, A. et al., Biol. Reprod., 1076-1083 (1994); Jayashankar, R. et al., Prostate, 14, 3-11 (1989)]; and three against chorionic gonadotropin (CG) [Jones, W.R. et al., Lancet, 8598, 1295-1298 (1988); Talwar, G.P. et al., Proc. Natl. Acad. Sci. USA, 91, 8532-8536 (1994); and Dirnhofer, S. et al., Immun. Today, 15, 469-474 (1994)].

The hormone hCG is an especially attractive target for immunological reproductive intervention for several reasons. First, the physiological chemistry of the hormone is well-known, being essential for the maintenance of pregnancy. Secondly, its amino acid sequence has been deduced, with a structure comprising a heterodimer of an α and β subunit. Each subunit is encoded by separate genes located on different chromosomes [Dirnhofer, S. et al., J. Endocrin., 141, 153-162 (1994)]. Thirdly, the immunological inactivation of hCG by circulating antibodies was shown not to significantly interfere with other physiological processes in the female, such as ovulation.

The physical characteristics of hCG, as mentioned, have been widely studied. This glycoprotein has a relative molecular mass (Mr) of about 38,000 which is divided into about 14,500 in α and about 22,000 in β. The α

subunit of hCG is identical in structure to the α subunits of the three pituitary hormones FSH, luteinizing hormone (LH), and thyroid-stimulating hormone (TSH). Furthermore, the α subunit has no hormone-specific properties though the β subunit is receptor-specific for target tissues. Because the α subunit of hCG is shared by other hormones, it follows that antisera raised to the entire hCG molecule can cross-react with other hormones, while antisera raised to only the β subunit of hCG exhibit much less cross-reactivity. These results have suggested that the β subunit of hCG would be useful as a highly specific immunological agent for the regulation of mammalian reproduction.

Three such βhCG-based immunogens have been tested. The World Health Organization (WHO) evaluated a βhCG carboxy-terminal peptide (βhCG-CTP) of 37 amino acids in length which is coupled to a diphtheria toxoid carrier in an adjuvant containing a synthetic muramyl dipeptide [Jones, W.R. et al., (1988) supra]. In this formulation, the use of a carrier such as diphtheria toxoid is required as the βhCG hormone is a "self" molecule normally not immunogenic in the human body. Preliminary results of this study indicated that antibodies raised against βhCG-CTP neutralize the biological activity of hCG in a mouse uterine weight gain assay [Stevens, V.C. et al., Am. J. Reprod. Immunol., 1, 307-314 (1981)]. A similar immunogen formulation incorporating βhCG-CTP was shown to be effective in reducing the fertility of a group of female baboons from about 70% to less than about 5% [Stevens, V.C. et al., Fertil. Steril., 36, 98-105 (1981)]. However, due to the fact that not all individuals mount an immune response to this formulation and even when a response is mounted, the antibody titer is quite low, WHO has placed these efforts on hold.

The second βhCG immunogen formulation now or previously in clinical trials was developed by the National Institute of Immunology, New Delhi, and consists of βhCG linked to the α subunit of ovine LH (oLH) attached to a diphtheria-tetanus toxoid carrier in a sodium phthalyl lipopolysaccharide adjuvant [Talwar, G.P. et al., supra (1994)]. Though it is

predicted that this immunological combination will be effective in neutralizing hCG, cross-reactivity with other hormones can occur due to the presence of the α subunit.

The third immunogen formulation now or previously in clinical trials has been developed by the Population Council, New York, and is based on the entire βhCG subunit [Tsong, Y. et al., 75th Annual Meeting Endocrine Society, Las Vegas, 282 (1993)]. The βhCG molecule is linked to a tetanus toxoid carrier in an aluminum oxide adjuvant. In this formulation, there is the potential problem of carrier-induced suppression of anti-hCG antibody production by the tetanus toxoid.

None of these prototype βhCG immunogen formulations are believed to be suitable for widespread commercial use. Primarily, the procedures used to obtain the βhCG either synthetically or from biological tissues/fluids are laborious, requiring a battery of purification steps which usually results in low yields, high manufacturing costs, and cross-contamination by other proteins, most notably αhCG.

Mukhopadhyay, et al., Am. J. Reprod. Immunol., 39, 172-182 (1998), described the production of an immunogenic βhCG by a recombinant DNA method. Mukhopadhyay and his associates injected rats and monkeys with the recombinant βhCG and an aluminum hydroxide adjuvant, either alone or conjugated to tetanus toxoid, to evaluate its immunogenic potency as a birth control vaccine. Although the βhCG alone evoked an immune response, conjugation of the recombinant βhCG with tetanus toxoid was required to increase the antibody titre.

One problem existing in the art is caused by the fact that βhCG is a "self" antigen and therefore, is not recognized by the immune system when injected, *i.e.*, the immune system fails to produce antibodies to the injected antigen. Therefore, a carrier, such as tetanus or diptheria toxoid is essential for generating an immune response. Attachment of such a carrier to the βhCG makes the βhCG more foreign and induces the immune system to

generate a stronger response. However, carrier induced suppression is a problem associated with the use of carriers to generate an immune response. In situations where the immune system of the recipient has been previously exposed to the carrier, the immune response to the carrier can be much greater than the immune response to the antigen linked to the carrier.

There are other unsolved problems in the art as well. For example, the adjuvants currently being used, such as synthetic muramyl dipeptide, sodium phthalyl lipopolysaccharide, or alum can produce side effects such as erythema, subcutaneous nodules, contact hypersensitivity, or granulomatous inflammation when administered. Therefore, a better tolerated adjuvant is desirable. In addition, some individuals simply fail to respond to the currently available vaccines and immunization regimes. A need for an immunization regime and properly formulated vaccines and which would effect an immune response to hCG in all treated individuals while avoiding the above described problems continues to exist in the art.

The present invention addresses these problems by providing a vaccine comprising a βhCG protein in a well-tolerated chitosan-based adjuvant which induces the production of anti-hCG antibodies in a host mammal and which immunization regimen overcomes the problem of non-responsiveness in some individuals.

SUMMARY OF THE INVENTION

The present invention provides improved compositions for inducing infertility in female mammals, including humans. According to the invention, induction of infertility is achieved by administration of an effective amount of the compositions preferably in an injectable formulation. The presently preferred injectable formulation comprises, in combination, βhCG protein and/or fusions, fragments or analogs thereof, and a chitosan-based adjuvant. In a preferred embodiment, the chitosan adjuvant comprises an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant and

an aqueous buffer, (see allowed U.S. Patent Application No. 08/823,143 incorporated herein by reference) wherein the ratio of βhCG protein and/or fusions, fragments or analogs thereof, to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w). A presently preferred biodegradable oil is squalene. In another preferred embodiment, the chitosan adjuvant comprises, in combination, chitosan, a metal salt and an aqueous buffer (see U.S. Patent Serial No. 5,912,000 incorporated herein by reference) wherein the ratio of βhCG protein and/or fusions, fragments or analogs thereof, to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w). The presently preferred metal salts are zinc acetate, nickel sulfate and copper sulfate. In another embodiment, the recombinant βhCG protein comprises a fusion protein wherein the βhCG protein or fragment or analog thereof is joined to any non-hCG polypeptide. In a presently preferred embodiment, the βhCG protein or fragment or analog thereof is joined to a β-galactosidase protein or fragment thereof in combination with any of the above described chitosan-based adjuvants.

The present invention is also directed to methods for inducing infertility in female mammals, including humans, by administering to the mammal a vaccine comprising βhCG protein and/or fusions, fragments or analogs thereof in combination with a chitosan-based adjuvant. The dose of the vaccine is such that it is effective to stimulate the production of antibodies in the mammal which recognize native circulating hCG proteins of the mammal and/or to prevent fertility. Preferred mammals include, but are not limited to, humans, dogs, cats, cows, horses, pigs, sheep, monkeys, rodents, elephants, and lagomorphs.

Proteins useful in the practice of the invention thus comprise βhCG protein and/or fusions, analogs and immunologically active fragments thereof which retain the ability to stimulate production of antibodies to hCG upon administration to a mammal. Such immunologically active fragments can be defined as containing at least one epitope effective to stimulate the

production of antibodies upon administration to a mammal in accordance with this invention or which is recognized by antibodies directed to hCG.

A preferred mode of administration of the vaccine is by intramuscular, intraperitoneal, or subcutaneous injection.

The present invention is also directed to a method for inducing infertility in a mammal by way of a cross-immunization regime wherein administration of a vaccine comprising a recombinant βhCG expressed in one species of host cell is followed by a second vaccine comprising a recombinant βhCG produced in a different species of host cell. Preferably, the vaccines are administered by injection. The sequence of injections is variable. Preferred expression systems used to express the βhCG include bacteria, yeast, baculovirus, and mammalian cells. Alternatively, the cross-immunization regime can be employed wherein each vaccine comprises a recombinant βhCG in combination with a chitosan-based adjuvant.

Another aspect of the invention is directed to methods for inducing antibody formation by the sequential administration of a recombinant antigen prepared in one expression system followed by the administration of the same or very similar recombinant protein antigen prepared in a second expression system. Preferred expression systems include bacteria, yeast, baculovirus, and mammalian expression systems. Preferred adjuvants include chitosan-based adjuvants.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to compositions comprising βhCG proteins in combination with chitosan-based adjuvants and their use in mammals in order to stimulate production of antibodies to the mammal's endogenous βhCG and to induce transient infertility. In addition, the compositions of the invention may be used in a cross-immunization regime to overcome the inability of some mammals to mount an immune response to certain antigens, particularly, self antigens.

The term "nucleotide sequence" refers to a heteropolymer of nucleotides or the sequence of these nucleotides. The terms "nucleic acid' and "polynucleotide" are also used interchangeably herein to refer to a heteropolymer of nucleotides. Generally, nucleic acid segments provided by this invention may be assembled from fragments of the genome and short oligonucleotide linkers, or from a series of oligonucleotides, or from individual nucleotides, to provide a synthetic nucleic acid which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon, or a eukaryotic gene.

The term "recombinant" when used herein to refer to a polypeptide or protein means that a polypeptide or protein is derived from recombinant (*e.g.*, microbial or mammalian) expression systems. "Microbial" refers to recombinant polypeptides or proteins made in bacterial or fungal (*e.g.*, yeast) expression systems. As a product, "recombinant microbial" defines a polypeptide or protein essentially free of native endogenous substances and unaccompanied by associated native glycosylation. Polypeptides or proteins expressed in most bacterial cultures, *e.g., E. coli*, will be free of glycosylation modifications; proteins expressed in yeast will, in general, have a glycosylation pattern different from those expressed in mammalian cells.

The term "recombinant expression vehicle or vector" refers to a plasmid or phage or virus or vector, for expressing a polypeptide from a DNA (RNA) sequence. An expression vehicle can comprise a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers; (2) a structural or coding sequence which is transcribed into mRNA and translated into protein; and (3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport

sequence, it may include an N-terminal methionine residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

The term "recombinant expression system" means host cells which have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit extrachromosomally. Recombinant expression systems as defined herein will express heterologous polypeptides or proteins upon induction of the regulatory elements linked to the DNA segment or synthetic gene to be expressed. This term also means host cells which have stably integrated a recombinant genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers. Recombinant expression systems as defined herein will express polypeptides or proteins endogenous to the cell upon induction of the regulatory elements linked to the endogenous DNA segment or gene to be expressed. The cells can be prokaryotic or eukaryotic.

The term "naturally occurring polypeptide" refers to polypeptides produced by cells that have not been genetically engineered and specifically contemplates various polypeptides arising from post-translational modifications of the polypeptide including, but not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation.

The term "analog" (or "variant") refers to any polypeptide differing from naturally occurring polypeptides by amino acid insertions, deletions, and substitutions, created using recombinant DNA techniques. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing activities of interest, such as the ability to stimulate the production of antibodies, may be found be comparing the sequence of the particular polypeptide with that of homologous human or other mammalian peptides and minimizing the number of amino acid sequence changes made in regions of high homology (conserved regions) or by replacing amino acids with consensus sequences.

Amino acid "substitutions" may be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, *i.e.,* conservative amino acid replacements. In addition, amino acid "substitutions" may be the result of replacing one amino acid with another amino acid whereby the activity of interest is increased, *i.e.*, non-conservative amino acid replacements.

"Conservative" amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Insertions or deletions are typically in the range of about 1 to 5 amino acids. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

The term "fragment" refers to a stretch of amino acid residues of at least about 5 amino acids, often at least about 7 amino acids, typically at least about 9-13 amino acids and in various embodiments at least about 17 or more amino acids.

The term "active" refers to those forms of the polypeptide which retain the biological and/or immunological activities of any naturally occurring polypeptide. According to the invention, the term "immunologically active" with reference to βhCG means that the polypeptide retains at least one of the immunological activities, preferably the ability to stimulate production of antibodies to hCG upon administration to a mammal. An immunologically active fragment comprises at least one epitope effective to stimulate the

production of antibodies upon administration to a mammal in accordance with this invention or which is recognized by antibodies directed to hCG.

The term "infection" refers to the introduction of nucleic acid into a suitable host cell by use of a virus or viral vector.

The term "transformation" means introducing DNA into a suitable host cell so that the DNA is replicable, either as an extrachromosomal element, or by chromosomal integration.

The term "transfection" refers to the taking up of an expression vector by a suitable host cell, whether or not any coding sequences are in fact expressed.

The polypeptides of the invention include, but are not limited to, a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or the amino acid sequence encoded by the cDNA insert of clone TOPP2 containing the pZ179 vector (SEQ ID NO: 1). Additional embodiments of the invention include, but are not limited to, a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 4 or the amino acid sequence encoded by the cDNA insert of the pZ500 vector set forth in SEQ ID NO: 3. Polypeptides of the invention also include active fragments or analogs of the βhCG protein sequence of SEQ ID NOs: 2 or 4. The polypeptides of the invention further embrace fusions or modifications of βhCG wherein the βhCG or analog is fused to another moiety or moieties to provide a more stable protein or to maximize expression levels.

Protein compositions of the present invention may further comprise a chitosan-based adjuvant. Preferred embodiments include a chitosan-based adjuvant comprising an emulsion of chitosan, sodium hydroxide, a biodegradable oil, a surfactant and an aqueous buffer as well as a chitosan-based adjuvant comprising chitosan, a metal salt and an aqueous buffer. The ratio of βhCG proteins and/or fusions, fragments or analogs thereof, to adjuvant is in the range of about 1:20 (w/w) to about 1:1500 (w/w). A preferred biodegradable oil is squalene. Preferred metal salts include, but are not limited to, zinc acetate, nickel sulfate, and copper sulfate.

A number of types of cells may act a suitable host cells for expression of the protein. Mammalian host cells include, for example, monkey COS cells, Chinese Hamster Ovary (CHO) cells, human kidney 293 cells, human epidermal A431 cells, human Colo205 cells, 3T3 cells, CV-1 cells, other transformed primate cell lines, normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, primary explants, HeLa cells, mouse L cells, BHK, HL-60, U937, HaK or Jurkat cells.

Alternatively, it may be possible to produce the protein in lower eukaryotes such as yeast or in prokaryotes such as bacteria. Suitable yeasts include *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces* strains, *Candida*, or any yeast capable of expressing heterologous proteins. Potentially suitable bacterial strains include *Escherichia coli, Bacillus subtilis, Salmonella typhimurium,* or any bacterial strain capable of expressing heterologous proteins. Preferably, the host cells or expression systems used to express the βhCG proteins and/or fusions, analogs or fragments thereof are of bacterial, yeast, mammalian, or baculoviral origin. Particularly preferred are yeast and bacterial host cells.

Therapeutic methods of inducing transient infertility in a female mammal by administration of βhCG proteins in combination with a chitosan-based adjuvant in order to produce antibodies to the mammals endogenous βhCG and/or to regulate the fertility of mammals is also contemplated. Although native proteins may be used, for present purposes, the source of βhCG used for immunization is preferably of recombinant origin. The use of recombinant proteins is advantageous because unlike naturally occurring proteins, they are economical to produce on a large scale.

The therapeutic methods of the invention include administering a vaccine comprising an effective amount of the polypeptides of the invention in combination with a chitosan-based adjuvant to a mammal thereby inducing transient infertility. Preferred modes of administration include, but are not limited to, intramuscular, intra peritoneal, or subcutaneous injection. The vaccine may be given as a single dose, however, two or more doses are

preferred. The vaccines may also be used to induce higher titers of anti-hCG antibodies than are otherwise possible.

The therapeutic methods of the invention further include a cross-immunization regime comprising a combination of βhCG vaccines. A vaccine comprising βhCG produced in one species of host cell is administered to the mammal. The initial vaccine is followed by administration of a vaccine comprising βhCG produced in a different species of host cell. This cross-immunization regime is useful to overcome the inability of some mammals to mount an immune response to certain antigens, particularly "self" antigens. In a preferred embodiment, a chitosan-based adjuvant is combined with the recombinant βhCG before administration.

Recombinant βhCG proteins and/or fusions, analogs or fragments thereof, may be obtained by methods well known in the art. Generally, the βhCG gene is inserted into a vector or plasmid which is then transformed or transfected into a host cell. Although not required, the coding sequence for a polyhistidine tag is added onto the βhCG gene in order to facilitate purification of the expressed protein. Other modification may also be made to the coding sequence to generate analogs, fragments and fusion proteins. The transformed host cells are then grown in appropriate medium and induced to produce the desired βhCG proteins, analogs or fragments thereof, and/or fusion proteins. The desired βhCG proteins are then isolated using chromatography. The common and usual techniques of modern molecular biology known in the art are used for vector construction, protein expression, and protein purification. A more detailed protocol is provided in the following non-limiting examples.

EXAMPLE 1

Bacterial Expression of βhCG Proteins

The bacterial expression of βhCG proteins was undertaken by constructing a vector, pZ179, comprising a DNA insert having the nucleotide sequence set forth in SEQ ID NO: 1. The vector pZ179 was derived from

pZ98 (Gupta, *et al*, *Biol. Reprod.*, 55:410-415 (1996) and carries the codons for the first 20 amino acids of β-galactosidase (β-gal). The sequence between the restriction sites SmaI and SalI was replaced with the DNA sequence for the βhCG codons for amino acids 22-165. An adapter DNA sequence carrying the restriction sites SmaI, PstI and BglII was added to the 5' end of the βhCG DNA fragment. This adapter sequence contains four codons that are not native to either β-gal or βhCG. These changes provided a more stable protein and also allowed expression levels of the recombinant protein to be maximized in *E. coli*. SEQ ID NO: 1 therefore codes for a βhCG/β-gal fusion protein consisting of leaderless βhCG linked to a β-gal fragment. The amino acid sequence of the encoded fusion protein is set forth in SEQ ID NO: 2.

Other changes to the native βhCG nucleotide sequence include the replacement of the stop codon of the native sequence with a SpeI restriction site in order to add a polyhistidine tag sequence at the carboxy terminal end of the protein. A pTAC promoter was used so expression could be induced by addition of the isopropyl β-D-thiogalactoside (IPTG). The host bacteria used for expression was TOPP2 (Stratagene). The pZ179 vector DNA was transformed into competent TOPP2 cells by standard methods. TOPP2 clones containing the pZ179 vector DNA (selected with ampicillin) were screened on a small scale to identify a clone having good expression.

The expression protocol entailed inoculating one liter of LB broth containing 100 mg/ml ampicillin with a pZ179/TOPP2 clone. The culture was grown in a shaking incubator at a temperature of about 30°C with a shaking speed of about 250 rpm, until an optical density of about 0.4 OD_{600} was attained. The expression of recombinant proteins was then induced by adding IPTG to a level of about 0.3 mM in the medium. Induction was allowed to proceed for approximately four hours after which time the cells were harvested by centrifugation.

In order to obtain the βhCG fusion protein, the cell pellet was resuspended to a concentration of about 1 g/6 ml in denaturing buffer which

contained about 6 M guanidine-HCl, 100 mM NaH_2PO_4, and 10 mM Tris at a pH of about 8. The cell paste suspension was then rocked at room temperature overnight. The cell debris was removed from the suspension by centrifugation at a speed of about 17,000 rpm at a temperature of about 4°C for about 20 minutes. The supernatant containing the desired βhCG fusion proteins was retained for purification.

Isolation of the βhCG/β-gal fusion protein was accomplished by passing the supernatant through a nickel column at a flow rate of approximately 10 ml per minute. Previously, chelating Sepharose fast flow resin (Pharmacia) had been prepared by washing the resin with water, pouring the resin into a column, and washing the column with approximately five bed volumes of 100 mM $NiSO_4$, followed by more water, then denaturing buffer. After passage of the supernatant containing the βhCG/β-gal fusion protein through the column, six washes in the order given below were performed to elute the pure βhCG/β-gal fusion protein. Fractions of the eluent were taken at each stage and analyzed at an ultraviolet wavelength of 280 nM.

The washes were:

1) 6 M urea + 20 mM Tris, pH 8
2) 6 M urea + 20 mM Tris + 25 mM imidazole, pH 8
3) 6 M urea + 20 mM Tris + 25 mM imidazole + 300 mM NaCl, pH 8
4) 6 M urea + 20 mM Tris + 300 mM NaCl, pH 8
5) 6 M urea + 20 mM Acetic acid + 300 mM NaCl, pH 6
6) 6 M urea + 20 mM Acetic acid + 300 mM NaCl, pH 3.9

The fractions (2-4) containing the desired βhCG/β-gal fusion protein were pooled, then dithiothreitol (DTT) was added to 10 mM concentration to prevent oxidation of the protein. The protein was desalted by loading the fractions onto a G-25 Sephadex (Pharmacia) column and washing with a buffer of 6 M urea + 50 mM Tris + 10 mM DTT, pH 8. The protein was analyzed by polyacrylamide gel electorphoresis (PAGE) using standard

techniques. The desalted protein was concentrated by loading onto a Sepharose-Q column (Pharmacia) in the same buffer and eluting with 100 mM NaCl. The purified βhCG/β-gal fusion protein was stored at -70°C.

EXAMPLE 2

Expression of βhCG Proteins in Yeast

The expression of βhCG in yeast was begun by cloning a 770 basepair BamHI fragment containing the βhCGB sequence fused with the alpha-mating factor leader sequence at the N-terminus into the BglII/BamHI sites of the yeast expression vector YEpFLAG-1 (Sigma). Next, the region of the vector from the unique NruI and SmaI sites was removed and the vector was religated deleting the DNA sequence coding for the FLAG peptide. The final vector was termed pZ500 and contains the DNA set forth in SEQ ID NO: 3. The amino acid sequence of the encoded protein is set forth in SEQ. ID NO: 4.

S. cerevisiae BJ3505 (Sigma) was transformed with pZ500 by combining 100μg salmon testes carrier DNA and 0.1 μg pZ500 expression vector DNA in a 1.5 ml microfuge tube. 100 μl of competent BJ3505 yeast cells were added to the microfuge tube and vortexed for five seconds. After vortexing, 600 μl of PLATE buffer (40% PEG 3350, 100 mM Lithium acetate, 10 mM Tris-HCL, 1 mM EDTA, pH 7.5) was added and vortexed. The microfuge tubes were then incubated at 30°C while shaking at 250 rpm on an orbital platform. 80 μl of DMSO was added and the cells were heat shocked at 42°C for 15 minutes. After centrifuging the cells for 3 seconds, the supernatant was removed and the cells were resuspended in 500 μl of sterile water. The transformed cells were then selected by growth on plates without tryptophan.

Yeast colonies expressing βhCG were grown in YPD medium until plateu phase (no further increase in optical density). The ADH-2 promoter used in the vector doesn't require addition of an inducing agent since it is activated upon carbon starvation which occurs when the carbon source in the culture medium has been depleted. After 48 hours, the cells are

removed by centrifugation and the supernatant is stored at -20° until purification.

βhCG was purified from the supernatant by adding two volumes of dilution buffer (30 mM NaOAc, 23 mM EDTA, pH 4.8). The dilute supernatant was passed through an SP column (Pharmicia) which had been pre-equilibrated with 10 column volumes of equilibration buffer (20 mM NaOAc, 40 mM NaCl, 15 mM EDTA, pH 4.8). 10 volumes of equilibration buffer was used to wash the column followed by washing with 10 volumes of wash buffer (20 mM NaOAc, 40 mM NaCl, pH 4.8). The protein was then eluted in 3-5 volumes of cobalt column binding buffer (50 mM NaH_2PO_4, 20 mM Tris, 0.1 M NaCl, pH 8.0). The resulting elution was passed through a cobalt column which was then washed with 10 volumes of binding buffer. The protein was then eluted with 2-3 volumes of binding buffer with 100 mM imidazole. Finally, the imidazole was removed by dialysis in PBS, pH 8.0 and the purified protein was stored at -20°C.

EXAMPLE 3

Epitope Mapping of βhCG

In order to identify epitopes in βhCG recognized by polyclonal antibodies directed to both bacterial and yeast expressed βhCG, a set of overlapping peptides was synthesized and spotted on a SPOTS membrane according to manufacture's directions with a kit from Genosys (The Woodlands, Texas). The synthesized peptides began at amino acid number 1 of βhCG as set out in SEQ ID NO: 2 and were each ten amino acids in length. Successive peptides had a three amino acid offset resulting in an overlap of seven amino acids between successive peptides. Membranes were then probed with rabbit polyclonal antisera produced against βhCG expressed in bacteria and in yeast. Epitopes recognized by the respective antisera were identified using methods well known in the art.

Results of the analyses revealed that both antisera recognized epitopes corresponding to amino acids 67-76 and 124-139 of βhCG. One of

the epitopes (67-76) resides within a receptor binding domain corresponding to amino acids 62-81 of βhCG. The other epitope (amino acids 124-139) overlaps with a C-terminal bio-neutralizing peptide corresponding to amino acids 135-169. The same methods have been used to identify other epitopes in βhCG that are recognized by antibodies to βhCG and to identify fragments of βhCG useful in the practice of the invention.

EXAMPLE 4

Preparation of βhCG Incorporated into a Chitosan / Oil Emulsion

A 2% chitosan solution in 0.5 M sodium acetate was prepared by dissolving 4.1 g of sodium acetate (Sigma Chemical Co., St. Louis, MO) in 50 ml of deionized (18 mOhm: dI) water with mixing. The pH of the solution was adjusted to 4.5 with approximately 7 ml of glacial acetic acid (Mallinkrodt Chemical, Paris, KY) and an additional 1.5 ml of glacial acetic acid was added to compensate for the effect of the addition of chitosan on the pH of the solution. The total volume of the solution was adjusted to 100 ml by the addition of dI water. About 2 grams of chitosan (Sigma Chemical Co., St. Louis, MO) was slowly added to the sodium acetate solution with stirring. The mixture was stirred for 2-3 hours to dissolve the chitosan. The chitosan solution was then sterilized by autoclaving. The autoclaved solution was cooled to room temperature in a biosafety cabinet. The chitosan solution was clarified by centrifugation in an IEC clinical centrifuge (International Equipment Co., Needham Hts., MA) at setting 7 for 5 minutes. The supernatant was decanted from the pellet which contained insoluble chitosan/chitin and contaminants. About 87 to 90% (by weight) of the chitosan was retained in the supernatant.

A 50% sodium hydroxide solution was prepared by dissolving 50 g of sodium hydroxide (Sigma Chemical Co., St. Louis, MO) in 100 ml of deionized water with mixing. A squalene/surfactant solution was prepared by combining 1500 µl of squalene (2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-

tetracosahexaene; Sigma Chemical Co., St. Louis, MO) with 600 µl of the surfactant Pluronic® L121 (BASF Corp., Parsippany, NJ) and vortexing until homogeneous.

Typically, chitosan/squalene/surfactant/antigen emulsions are prepared by adding approximately 420 µl of antigen in water or urea to approximately 370 µl of 2% chitosan in 0.5 M sodium acetate with vortexing. The actual amount of antigen used can range from less than 1 µg to several milligrams. About 10 µl of the 50% sodium hydroxide was added to the antigen/chitosan, and the mixture was vortexed. Aliquots of approximately 10 µl of 50% sodium hydroxide were added until a stable cloudy precipitate formed. Approximately 140 µl of the previously prepared squalene/surfactant solution was added to the above solution of antigen-chitosan. The resulting emulsion was vortexed for several minutes. Immediately prior to administration in the immunization studies, the emulsion of chitosan-antigen was mixed again by vortexing or syringe aspiration.

EXAMPLE 5

Preparation of Metal/Chitosan/Antigen Complexes Containing Either Zinc, Copper, or Nickel

To prepare chitosan/metal complex adjuvants containing either zinc, copper or nickel, a 2% chitosan solution was initially prepared by dissolving 2 g chitosan (CTC Organics, Atlanta, GA) in 100 ml 2% acetic acid. The resulting solution was sterilized by autoclaving. As an alternative, the chitosan solution can also be prepared by dissolving 2 g in 100 ml 0.5 M sodium acetate pH 4.5. A zinc acetate, nickel sulfate, or copper sulfate solution was prepared in deionized water at a molarity between 0.001 to 0.2 M and filter sterilized. The 2% chitosan solution was diluted 1:1 using deionized water and 4 ml of the resulting 1% chitosan solution was added to 10 ml of the desired metal salt solution. The resulting suspension was mixed on an end to end shaker for 2 to 4 hours at room temperature. The mixture was sonicated using a Branson Sonifier 250 for 3 to 5 minutes and the pH of the mixture

adjusted to 12.0 - 12.5 with 10 N NaOH during sonication. When the zinc salt was employed, a white precipitate was formed, when the nickel salt was used, the precipitate was light green, and when the copper salt was used, the precipitate was blue. After sonication, the mixture was centrifuged at 2000 rpm (1000 x *g*) for 10 minutes and supernatant discarded. The pellet containing the chitosan chelate was washed twice with PBS, pH 7.2, and centrifuged after each wash. The wet weight of the pellet was determined and the pellet was resuspended in 8 M urea, pH 7.8 to 8.0. The metal/chitosan complexes were stored in either 8 M urea or PBS at room temperature. The stored metal/chitosan complexes have shown to be stable for up to six months.

Antigens were associated with the metal/chitosan complex by the following procedure: recombinant protein containing six histidines was equilibrated in 8 M urea, then incubated with the chitosan metal complex at a ratio of about 100:1 in a plastic tube for 1 to 3 hours at room temperature. Following incubation, protein/metal/chitosan complex was pelleted by centrifugation for 10 minutes at 1000 x *g*. The amount of bound protein was estimated by determining protein concentration in the supernatant remaining after centrifugation and subtracting this amount from the amount initially added to the binding reaction. In general, the resulting ratio of antigen:metal/chitosan was about 1:500 (2 mg/1g, wet wt.). This chitosan-protein pellet was washed two times with PBS, resuspended in PBS and the concentration adjusted to 1 mg antigen/ml buffer for injection as described below.

EXAMPLE 6

Administration of Recombinant βhCG to Female Mice

Female mice were immunized by intra peritoneal injection with bacterial and yeast recombinant βhCG proteins produced by the methods described in Examples 1 and 2 in adjuvant described in Example 5. Prior to the initial immunization, animals were bled to obtain control serum. After the

initial immunization, the animals were bled at desired intervals. The titer of induced antibodies produced against βhCG was measured by ELISA, by methods well known in the art. The antibodies were then evaluated for the ability to neutralize the bioactivity of injected native hCG.

More specifically, ten mice were injected 3 times each at 3 week intervals with 25 µg of purified yeast-expressed βhCG in an adjuvant comprising chitosan and zinc acetate prepared as described above in a total volume of 100 µl. Serum antibody titers to native hCG measured by ELISA one month after the last of the three vaccinations are shown below.

Mouse number	Titer
161-1	16000
161-2	2000
161-3	32000
161-4	16000
161-5	8000
80-1	4000
80-2	8000
80-3	<250
80-4	8000
80-5	<250

Mice 80-3 and 80-5 initially did not mount an immune response to this vaccine containing βhCG expressed from yeast. Consequently, these mice were immunized two weeks later with 25 µg of a vaccine containing bacterially expressed βhCG. This cross-immunization resulted in a titer against native hCG of 4000 as measured by ELISA three weeks post vaccination. This rose to 12800 by six weeks post vaccination. These results demonstrate that animals which are non-responsive to yeast βhCG vaccine are induced to mount an immune response by cross-immunizing with a vaccine comprising bacterially expressed βhCG.

The data presented above shows that animals unable to mount an antibody response to a vaccine prepared in yeast were able to produce sufficient antibody titres against βhCG after administration of the same or a very similar antigen produced by a different species of host cell.

EXAMPLE 7

Administration of βhCG to humans

Although the foregoing examples describe administration of βhCG to mice the compositions and methods of the invention are also useful in other mammals, including humans. The proper dose of active agents for administration to humans may be readily determined by one of ordinary skill in the art.

An effective amount of the compositions of the invention are administered to humans to induce infertility or to stimulate production of antibodies to βhCG by intramuscular, subcutaneous injection or by other methods well known in the art.

In human subjects failing to mount an immune response after the initial administration of the compositions of the invention, the cross-immunization regime is employed. An effective amount of βhCG produced in a different species of host cell is administered by intramuscular or subcutaneous injection. In an alternative embodiment, the cross immunization protocol could be used as the first protocol.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and compositions and methods which are functionally equivalent are within the scope of the invention. Indeed, numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the present preferred embodiments. Consequently, the only limitations which should be placed upon the scope of the invention are those which appear in the appended claims.